Chapter 1

Assessing the ecological role of *PHYTOCHROME*, *CRYPTOCHROME* and *UV-B RESISTANCE 8* photoreceptor families in natural conditions: Life history traits depend on functional light responses

Abstract

Sunlight is an important abiotic cue and is perceived by different photoreceptors. These possess a wide range of functions in plant response to environmental stimuli. One central role is the regulation of flower onset, and therefore they influence plant reproductive fitness. The knowledge of photoreceptor functions is derived from lab based experiments, and not from studies under natural sunlight. This raises the question which effects may be expected *in natura* from plants defective in specific photoreceptors.

We tested responses to different natural environments by growing allelic null-mutants of *Arabidopsis thaliana*. Four major photoreceptors, *PHYTOCHROME A* and *B*, *CRYPTOCHROME 1* and *2*, as well as one central transcription factor in light response, *ELONGATED HYPOCOTYL 5* and a direct effector of flowering, *FLOWERING LOCUS T* were included in this study. These mutant lines were grown in two different seasons in the course of two years.

We show that one photoreceptor in particular, *PHYB*, has a strong effect on reproductive success, and confirm that many of the phenotypes described in the lab are also found in natural conditions, but not always to the same extent. Additionally we show that the effect size varies with natural conditions, and that spring and winter cohort fitness may depend on different photoreceptor interactions.

To summarize, this study contributes valuable and previously unreported ecological functions of photoreceptors under natural light conditions.

Introduction

Life revolves around the central life stage: reproduction. It is of the utmost importance for plants to sense environmental cues to flower at the optimal time, particularly for an annual such as *Arabidopsis thaliana* which has only one reproductive phase per generation. The most influential environmental factors for flower onset include temperature and light. The latter is important in multiple ways, *i.e.* day length, light intensity, and its composition; that is different wavelengths reaching the plant. Solar radiation triggers many different developmental, adaptive, defensive or stress responses (Björn 2015).

Light signals are sensed by different receptors, a total of 13 photoreceptors belonging to distinct families are known in *A. thaliana* (Kong & Okajima 2016). The *PHYTOCHROME* family responds to light in red to far-red spectrum (*PHYA-E*, involved in many developmental processes). Three different photoreceptor families respond to UVA/blue light: *CRYPTOCHROMES* (*CRY1*, *CRY2*), *PHOTOTROPINS* (*PHOT1*, *PHOT2*, active in blue light, responsible for phototropism) and *ZEITLUPE* family genes (*ZTL*, *FKF* and *LKP2*, co-acting on regulation of circadian clock). Finally, the unique *UVB* photoreceptor *UVR8* (discussed in Chapter 2) completes the list of known light sensory proteins in this model plant (but see Oakenfull and Davis (2017)).

Phytochromes are among the earliest receptor systems and a total of five different phytochromes are established in higher plants due to multiple gene duplication events (Rensing *et al.* 2016). Phytochromes respond to red and far-red light and play a vital role in germination time. Consequently they may affect reproductive success (Strasser *et al.* 2010; Donohue *et al.* 2012) by regulating physiological and developmental processes (but see Kaiserli and Chory (2016) and Ballaré (2017)). Cryptochromes share a common ancestry with DNA repair enzymes known as photolyases, but did obtain new roles as UVA/blue light photoreceptor. *CRY1* and *CRY2* therefore share several functions: They act on stomatal opening, root elongation and entrain the circadian clock (Chaves *et al.* 2011) and (Liu *et al.* 2011).

Our study focuses on four photoreceptors, *PHYTOCHROME A* (*PHYA*) and *B* (*PHYB*), and *CRY1* and *CRY2*, as they play a crucial role in plants and the different mutant lines are readily available at the Nottingham Arabidopsis Stock Centre (NASC, Liverpool, UK).

The functions of *PHYA* and *PHYB* are manifold and due to dissimilar photostability they are utilized differently. *PHYB* is the main receptor for red light and for sensing canopy shade, whereas *PHYA* is responsible for broader responses to far red and overall low intensity light, regardless of wavelength (Li *et al.* 2011). *PHYA* reportedly has effects on phototropism (Sullivan *et al.* 2016) and affects flowering time in long photoperiods (Sandhu *et al.* 2012). *PHYA* and *PHYB* also have potentially antagonistic effects on flowering: *PHYB* targets *CONSTANS* (*CO*) for degradation, whereas *PHYA*

functions in stabilizing *CO* (Greenham & McClung 2015). Furthermore a growth-defense tradeoff between Jasmonic Acid (JA) and *PHYB* signaling was found (Campos *et al.* 2016). *PHYB* is also temperature sensitive and therefore has a dual receptor function (Jung *et al.* 2016; Legris *et al.* 2016), while crosstalk of *PHYA* with water deficit (Auge *et al.* 2012) and wounding responses (Robson *et al.* 2010) was found. Additionally, recent investigations found that *PHYA* and *PHYB* do affect the primary plant metabolism (Han *et al.* 2017).

Functions for cryptochromes are similarly diverse. Importantly, *CRY1* is active under daylight conditions while *CRY2* is quickly degraded (Ahmad 2016). This is translated into unique roles of the cryptochromes in a similar fashion to *PHYA* and *PHYB*. *CRY1* regulates photmorphogenic responses to blue light and UVA radiation (Galvão & Fankhauser 2015; Su *et al.* 2017), and *CRY2* acts in low blue light influences flower onset (El-Assal 2003) by stabilizing *CO* (Turck *et al.* 2008). Cryptochromes also have several unique and unexpected qualities: magnetosensitivity (Ahmad *et al.* 2006; Maeda *et al.* 2012) and redox function in FADH-/FADH. The latter consequently affects reactive oxygen species (ROS) levels in a blue-light dependent manner, which may cause plant hardening (Jourdan *et al.* 2015; El-Esawi *et al.* 2017).

Interest in photoreceptor interaction has only recently gained traction with photobiologists. For example, a large number of signaling molecules downstream of *PHY* and *CRY* were found to be associated with both types of photoreceptors (Pedmale *et al.* 2016; Wang *et al.* 2018). This brings evidence for crosstalk between different light receptors and the potential co-dependence in stress response. My thesis expands on this in Chapter 2 where I describe an interaction between photoreceptors *UVR8* and *CRY1*. Importantly, *CRY* and *PHY* do jointly affect the circadian clock (Jarillo *et al.* 2001) and flowering as discussed earlier. Such overlaps may hinder effects caused by a gene-defect to produce a phenotype, because it may be masked by epistasis.

The main aims of this study were to investigate important traits for fitness and phenology in the field. Survival, silique number and flower phenology in mutant genotypes with alleles defective in photoreceptors (*PHYA*, *PHYB*, *CRY1*, *CRY2, UVR8*), *ELONGATED HYPOCOTYL 5* (*HY5*), a central transcription factor in response to light (Oyama *et al.* 1997; Ulm *et al.* 2004) and *FLOWERING LOCUS T* (*FT*) were measured (Table 1). We further investigated different life cycles prevalent in *A. thaliana* by growing mutants in the field in two consecutive winter and one spring season.

The questions we could answer herein are:
Are lab-reported phenotypes also found *in natura*? Are these bound to certain environments or are they ubiquitous? What are the fitness effects of photoreceptor mutants *in natura*? Are all photoreceptors essential for survival and reproduction, *i.e*. do we see phenotypes in all mutants when growing in the field? Do all allelic variants of a non-functional gene behave similarly?

Material and methods

Selected genotypes

All experiments included mutant lines in Landsberg *erecta* (L*er*) background . The tested mutant genotypes were ordered from NASC (www.nasc.co.uk), with the exception of *uvr8-1* and *hy5-1* which were provided by the Ulm laboratory (Geneva, Switzerland).

NASC ID	Gene	Allele / Name	Mutation	Source	mutagen	wild type
N20	-	*Landsberg erecta*	-		-	.
N6955	*CRY1_GL1*	*hy4-B104/gl1-1*	452bp deletion in *CRY1*; 10kb deletion in *GL1*	Oppenheimer *et al.* (1991); Bruggemann *et al.* (1996)	fast neutrons	Col-0
NA	*CRY1_CRY2*	*hy4-223_fha1*	300bp deletion in 3' region of *CRY1*	Vandenbussche *et al.* (2007)	-	L*er*
N186	*CRY2*	*fha-1*	W54 ➔ STOP	Koornneef *et al.* (1991)	EMS	L*er*-0
N187	*CRY2*	*fha-2*	G254 ➔ R	Koornneef *et al.* (1991)	x-ray	L*er*-0
N184	*FT*	*ft-2*	W138 ➔ STOP	Koornneef *et al.* (1991); Kardailsky (1999)	EMS	L*er*-0
N185	*FT*	*ft-3*	R119➔H	Koornneef *et al.* (1991); Kardailsky (1999)	EMS	L*er*-0
NA	*HY5*	*hy5-1*	Q4➔STOP	Koornneef *et al.* (1980); Oyama *et al.* (1997)	EMS	L*er*
N6219	*PHYA*	*phyA-201*	Q980 ➔ STOP	Nagatani *et al.* (1993)	EMS	L*er*
N6222	*PHYA*	*phyA-205*	V631 ➔ M	Reed *et al.* (1993)	EMS	L*er*
N6211	*PHYB*	*phyB-1*	Q448 ➔ STOP	Koornneef *et al.* (1980); Somers *et al.* (1991); Reed *et al.* (1993)	EMS	L*er*
N6213	*PHYB*	*phyB-5*	W552 ➔ STOP	Reed *et al.* (1993)	EMS	L*er*
N6216	*PHYB*	*phyB-8*	*unknown*	Somers *et al.* (1991); Reed *et al.* (1993)	EMS	L*er*
NA	*UVR8*	*uvr8-1*	15bp deletion	Kliebenstein (2002)	fast neutrons	L*er*

Table 1: Genotypes used in this experiment with NASC stock ID and mutagen description. Mutagen EMS = ethyl methyl sulfonate.

Plant material and growth conditions

We transferred pregrown plants to different environments, *i.e.* common gardens in Zurich ("Irchel Garden", 47°23'46.1" N, 8°33'04" E, 500m) in three consecutive seasons and a high elevation site in Graubünden (46°53'16.1" N, 9°29'21.6" E, 2000m) during one spring season. Different setups with multiple genotypes were used to investigate the effect of genes on plant survival and reproductive traits in field environments. We refer to *Appendix* I which describes date of transplant, transferred lines, conducted measurements and number of replicates for each measured trait.

Plant pretreatment

Seeds were surface sterilized with chloric gas (using HCl and NaClO) for four hours and then left in a fume hood for four hours before they were sown on 0.8% Agar plates with 1/2 Murashige & Skoog medium. After 72 h at 4 °C in the dark for stratification, plates were transferred to growth cabinets

(Sanyo) with a light period of 20 h, (22°C, 70% rH) and a dark period of 4 h (20 °C, 60% rH). Germinated plants were then transferred to biodegradable pots (Jiffy, 2.5x2.5x4cm) in soil (Einheitserde) and kept in a growth chamber (Kälte 3000) with long day conditions until plants reached a six leaf stage. The long day conditions comprised a 16 h light period (20 °C, 60% rH) and a dark period of 8 h (18 °C, rH 60%,120-160 µE light intensity)

Biodegradable pots were placed in two to four compartments in a complete randomization fashion per compartment, with at least four replicates per compartment. Each compartment at Irchel garden had dimensions of 1 m^2 with a 10 cm soil layer (Rasenerde Top-Dressing) enclosed by a slug barrier. Compartments at 2000 m in Graubünden had dimensions of 1 m x 2 m, with a layer of fleece and chicken wire at the bottom of the added soil layer. Pots were arranged at least 10 cm from the edges of the compartments and distributed with a gap between pots of at least three centimeters to allow solitary growth of each individual without touching other plants.

Measurements

We collected data in a similar fashion over the course of each experiment: flowering stage and survival were measured jointly at multiple points in time. Flowering stage was documented as "1" if bolting had occurred, "2" if a flower was open, "3" if silique were visible and "4" for plants with ripe siliques. We used the stage of bolting for further investigating the time to flower, while other stages were excluded from our analysis due to the inaccurate definition of these. Survival was recorded as either alive (1) or dead (0). Reproductive success was either measured during the plants life cycle by assessing the number of flower heads before siliques were visible or after harvest, by counting the total silique number of individuals.

Statistical analysis

Each experiment was analysed independently. To reveal the effect of mutant lines on the measured traits the term "class" for each of the nonfunctional genes as well as wild types was defined. For an overall analysis of all the traits, we generally used a one-way ANOVA (measured trait explained by class) on the most appropriate model implemented using R standard packages Hmisc, MASS, stats, as well as the additional package lme4 (Bates *et al.* 2014). When linear models were not applicable, we used generalized linear models fitted to poisson or binomial distribution. The most appropriate model was chosen by testing for normality, homoscedacity, overdispersion and comparison of applicable models with and without block effect using ANOVA (analysis of variance). A goodness-of-fit test was performed on generalized linear models.

Posthoc tests to specify differences between classes were conducted using a Tukey-test to a Simultaneous Tests for General Linear Hypotheses as implemented in the multcomp (Bretz *et al.* 2011) or "Tukeys Honest Significant Difference" method (TukeyHSD, R-package stats). When

models failed to converge or meet assumptions Kruskal-Wallis non parametric tests including Dunn's test for posthoc comparisons of classes were used. Subsets for the *PHYB* class were made to compare the three single-mutant lines included. Fishers exact test was performed in case of low number of replicates in binomial data.

Results

Global comparison

The first analysis aimed to identify the factors that affect plant flower time and fitness components overall; whether it was only the genotype or if environmental effects would contribute as well. In addition it was of interest to us if there would be an interaction effect, *i.e*. if certain genotypes would behave differently in the seasons or different fields (Table 2).

	Global comparison				
	Class	Environment	Survival	Flower onset	Interaction[2]
Time to flower	32.61 ***	1574.64 ***	-	-	14.57
Siliques	44.435 ***	1.069[1]	21.994 ***	0.572	NA
Survival	16.279 *	18.99 ***	-	-	9.47

[1] only season winter 2 and spring 1 in this comparison

[2] Class x Environment (GxE)

Table 2: ANOVA of model y~ Class*Environment+(survival or flower onset), with response term y in the left column. Numbers given are Chi-squared values with asterisks indicating significances (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). Class is significantly affecting all response terms.

Global analysis revealed that **time to flower** (Figure 1) is affected by class but also environment. Specifically, when we consider the day of transplant as the initial date, spring germinated plants flower significantly earlier than overwintering plants. Spring flowering started as early as three weeks after transfer of seedlings to the field, while in winter at least 14 weeks of vegetative growth were recorded. Flower onset in high elevations was significantly slower than at the Zurich field site. Floral organs were fully developed in a majority of individuals only after 12 weeks at the high elevation site, while the flowering phase was conceded (top flowers produced siliques) five weeks after transplant at the Zurich common garden (low site).

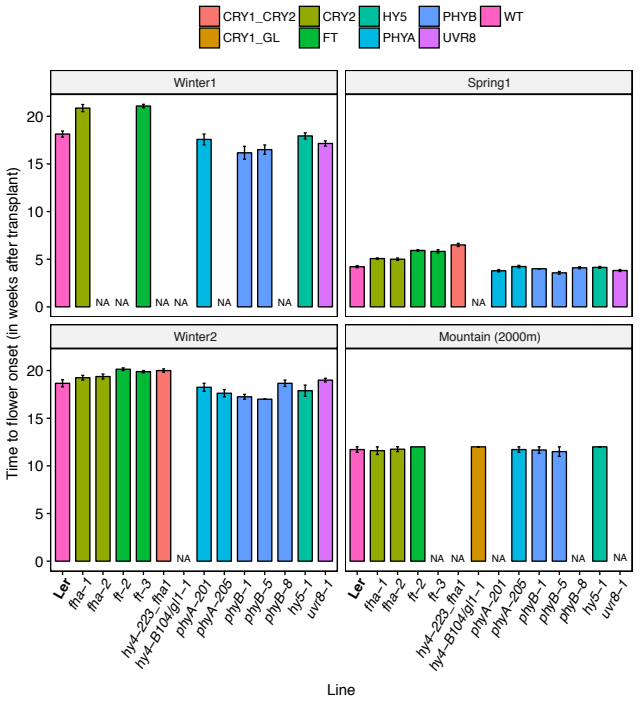

Figure 1: Bar plot of time to flower in weeks (error bars ±1 SE). Four boxes are shown, respective of the four transplant experiments, left boxes are winter season, right boxes are spring season. Colors indicate impaired gene functions, WT= wild type.

Silique number (Figure 2) is strongly affected by class and survival. This is unsurprising since we assigned zero siliques to dead plants. If only surviving plants are tested for silique number, then class alone is significant. We therefore conducted two different analyses in the "local" analysis including or excluding dead plants. The significance of class once again shows the strong effect of single genes on reproductive success. Interestingly, *phyB* always has reduced silique number while other mutants do not show a similarly extreme phenotype.

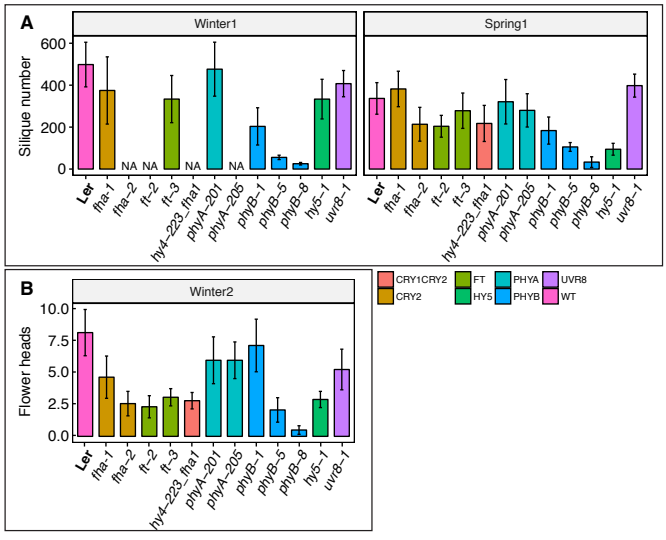

Figure 2: Bar plot with error bars (error bars ±1 SE) of produced reproductive units, *i.e.* siliques (2A; Winter 1, Spring 1) or flower heads (2B; Winter2). Colors indicate impaired gene functions, WT= wild type. A reduced silique count is visible in *phyB*, in spring *hy5* is also showing a low silique number.

Finally, we found that **survival** is affected by both class and environment.

Local comparison - by environment:

An analysis by environment was performed in separate subsets of the data, with "class" as the explanatory value for different traits. Class consisted of groups of mutants for each gene of interest (Table 3).

Influence of class by environment				
Environment	Winter 1	Winter 2	Spring 1	Mountain
Reproduction[†]	24.939 ***	14.057 .	17.436 *	NA
Flower onset	58.911 ***	53.894 ***	122.82 ***	4.765
Survival	13.397 *	3.196	2.32	5.238

[†] silique number in Winter 1 and Spring 1,
flower heads in Winter 2

Table 3: Statistical effect of class on response terms in different environments of this study. Numbers indicate Chi-squared values, asterisks refer to significances (. p<0.1, * p<0.05, ** p<0.01, *** p<0.001). One-way ANOVA (y~class) was performed.

Winter 1

We found significant effects of class on **silique number** (p<0.001). In this season *phyB* had a reduced silique number in comparison with other lines. Here we found two of three *PHYB* mutants (*phyB-5* and *phyB-8*) had significantly less fruits than the wild type (Figure 3).

Flowering onset was significantly different (p<0.001). This was effectively an influence of three classes: *phyB* had an early flower phenotype (p<0.005) while *ft* (p<0.005) and *cry2* (p<0.005) had a later flower onset.

Survival was significantly lower in *phyB-8* (p=0.013). No other significant relationships were detected, despite *phyB* consistently showing a distinct and weakened phenotype in their vegetative growth (etioplasts leading to light-green leaves).

Winter 2

In this season we collected information on reproduction rate by measuring the number of inflorescence heads when the first flowers were already open. We further measured survival and flower time.

The reproduction rate, here measured as **flower heads per plant,** showed an almost significant class effect (p=0.05018). Posthoc analysis found that early flowering phenotypes of *phyB* (p=0.013) as well as late flowering *ft* (p<0.01), *cry1cry2* (p=0.017) produced a reduced number of flower heads. We also found a significant reduction of flower heads in *cry2* (p=0.015) and *hy5* (p=0.037), neither of which show flowering time differences. Late and early flowering show significant phenotypes. However, these results should be interpreted with care, because flower head count in late flowering genotypes

may not necessarily reflect the final number of fruits produced but may be an artifact of a one-time measurement.

A significant effect of class on **flower onset** was found (p=1.7e-09). Consistent with Winter1 results, we found that *phyB* (p=0.0219) is early flowering while *ft* (p=0.0026) and *cry1 cry2* (p=0.0055) are late flowering. In contrast to results from the previous year, *cry2* did not have a phenotype in this environmental condition.

We found no effect on **survival** for class, but a strong block effect was detected. This effect was clearly visible in the field: one of four compartments showed a very high mortality rate, while the other three had more survivors regardless of genotype.

Spring 1

We saw an overall earlier flower onset than in winter. Only four weeks after transplant, fully flowering individuals were found, while flowering occurred only after more than 13 weeks in the overwintering plants,.

We found a difference in **silique production** among classes (p=0.0148), *phyB* had a strong reduction (p<0.001) in silique number, while *hy5* also produced significantly fewer siliques than the wild type (p<0.001). This difference was retained when including only live plants(*phyB*: p<0.01, *hy5*: p=0.012). In *phyB*, the mutants once more showed disparities in this trait, with *phyB-8* as the least fit (*phyB-8* < *phyB-1*, p=0.011, Figure 3).

Flower time was again affected by class (p<0.001). However, *phyB* did not harbor an early flowering phenotype during spring this season. Only late flower onset phenotypes in *ft*, *cry2* and *cry1cry2* could be found (all p<0.001). We detected that *cry1cry2* mutants had the latest flower onset, followed by *ft* and *cry2*.

Survival was consistently high among all classes, which may be attributed to the short life cycle that was common in spring.

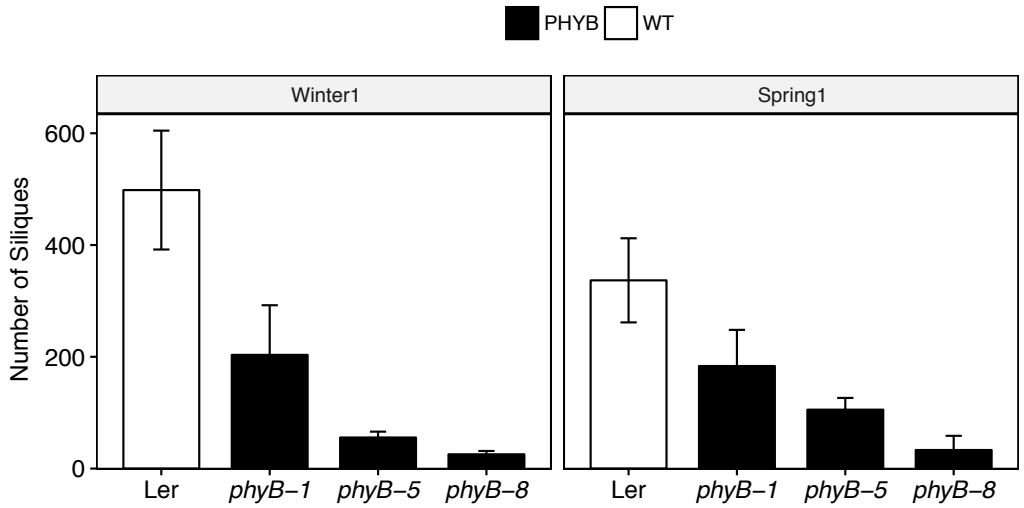

Figure 3: Bar plot of silique number of Winter 1 and Spring 1 showing L*er* and three *phyB* alleles. A significant difference between *phyB-1* and *phyB-8* was found in both environments, and *phyB-1* has no significant differences to L*er*. (Error bars indicate ±1SE.)

Mountain (2000m)

We transplanted seedlings to a high elevation site in late spring 2014 to test whether potential contrasts in plant fitness and flower time were detectable. However, due to herbivore damage to floral organs, silique number was not measured. Thus, we recorded survival and flower time (Figure 1). Flower time was only measured two times since the site was not easily accessible.

We found an increase in mortality for *uvr8* and *cry1 gl1* (p=0.0254). In addition *phyB* showed a high mortality that was close to significantly different to the wild type (p=0.0598).

Discussion

Testing gene functions in a naturally fluctuating environment is fundamentally challenging. Many unknown or immeasurable environmental factors affect an individual in field experiments (Prasch & Sonnewald 2015). Yet these experiments may reveal genotype-environment (GxE) effects as well as corroborate function in nature and may even elucidate epistatic effects that would prohibit a phenotype from occurring in certain environments (Tonsor *et al.* 2005; Kudoh 2016).

Non-functional variants of genes can be ordered online (NASC, ABRC) for *A. thaliana*, yet many have no clear phenotype assigned to them in these stock centers. We selected photoreceptor mutants for their central role in plant development and environmental response. Even though much is known about photoreceptors such as Phytochromes (Kaiserli & Chory 2016) and Cryptochromes (Chaves *et al.* 2011), it is worth noting that the vast majority of studies on these were conducted in laboratory environments. Thus, an investigation of the field-responses (including phenotypes from multiple field seasons) of these often tested genotypes adds value to the current state of knowledge.

What are the fitness effects of photoreceptor mutants* in natura*? Are all photoreceptors essential for survival and reproduction,* i.e. *do we see differences to wild types in the tested mutants?

Our results showed that reproductive success may depend on a correct perception of the photoperiod and light quality, and that certain photoreceptors may have a larger effect on fitness. We highlight that *phyB* showed a significant reduction in fruit production as a fitness component in all environments, thus defects in this gene principally lead to a loss-of-fitness in winter and spring.

Our results show that overall the difference in environmental conditions strongly affects the flower onset and fitness of plants, but we mostly found no interactions of genotype and environment. This clearly supports the importance of photoreceptors.

The data presented in this chapter also highlights the effect of seasonality on flower onset as well as reproductive success: winter cohorts have more siliques but a longer generation time while in spring this pattern is inverted.

Do all allelic variants of a non-functional gene behave similarly?

We detected strong differences in the fitness of allelic variants of *phyB*. All three alleles included in this study are described as strong alleles and show no accumulation of *PHYB* proteins (Reed *et al.* 1993). The *phyB-1* genotype in this study was similar to the wild type, despite being a strong null-mutant similar to *phyB-5* and *phyB-8* (Reed *et al.* 1993). We speculate that the difference in the origin of the mutants *phyB-1* (Koornneef *et al.* 1991) to *phyB-5* and *phyB-8* (Reed *et al.* 1993) may have led to this discrepancy. Further experiments should therefore include genotypes and wild types of one source, and include molecular fingerprinting and protein accumulation tests of included mutant lines to confirm the correct genotype is in use.

Are lab-reported phenotypes also found* in natura*? Are these bound to certain environments or do they always occur?

The observed **flower phenology** was mostly in concordance with current knowledge about photoreceptor effects in control conditions. Many phenotypes that we found in the field did corroborate previous findings of late flowering in *ft* and *co* (Koornneef *et al.* 1991) and *cry1 cry2* (Mockler *et al.* 1999) and early flowering in *phyB* (Guo 1998). In *cry2* the late flower onset was not found in all environments (Guo 1998), potentially showing that *CRY2* function may be compensated for by other genes (e.g. *PHYA* and *FKF*) acting on floral induction (Song *et al.* 2015). Another potential effect that may be drawn from this observation is that because Cryptochromes and Phytochromes directly interact with the same array of genes (Wang *et al.* 2018) a null-mutation in one gene may allow other photoreceptors to increase their regulatory function (Su *et al.* 2017).

Silique number had no consistent consequences for fitness, despite the broad range of functions lacking in the photoreceptor mutants (Oakenfull & Davis 2017). The exception was *phyB*, which

produces a strikingly strong phenotype in the field, consistent with other lab based results (Ballare *et al.* 1997; Mani & Guruprasad 2015). Only few siliques were produced and overall a stunted growth with few and small leaves were observed for two of the three allelic variants in *phyB*.

Survival was found to be mostly similar among genotypes (exception: *phyB-8* in winter 2) and varied between the different environments. The exception was survival at 2000m, where *uvr8* and *cry1 gl1* show high mortality. This may be explained by increased solar radiation at higher elevations (Blumthaler 2012) and therefore photomorphogenic responses such as anthocyanin accumulation triggered by *CRY1* (Chaves *et al.* 2011) and especially *UVR8* (Yin & Ulm 2017) may be essential.

In addition, several new phenotypic differences were found in our experiments.
Instead of a reported late flowering phenotype in *phyA* in long photoperiods (Sandhu *et al.* 2012) we found *phyA* and wild types were similar in Spring 1. In general this mutant did not show any phenotype in our study. This is a surprising result considering that our experiments were performed under shorter (Winter 1, Winter 2) and longer (Spring 1) photoperiods. However, more experiments in different photoperiod conditions with a larger number of replicates may be necessary to confirm these findings.
An early flower onset for *phyB* which has been reported previously for warmer temperatures in climate controlled chambers (Halliday *et al.* 2003) could be confirmed by our study. This early flower onset was found only when plants were overwintering but not in the spring cohort, even though it was reported by the same study that at lower temperature (16°C) flowering is similar to wild type plants. We note that, because we effectively grew *phyB* in only a single spring cohort, and flowering time is almost significantly earlier in these mutants ($p=0.0748$), more experiments in field conditions are needed to fully corroborate our finding.
Furthermore we found inconclusive results in *hy5-1*. In winter, mostly no effect could be detected, with exception of flower head reduction, but as mentioned before these results have to be interpreted with caution. This may be caused by *HYH* (*HY5 HOMOLOG*), which has a potentially redundant function to *HY5* (Holm 2002; Brown & Jenkins 2008) and CO, which has a joint function with *HY5* in promoting de-etiolation and flowering (Galvão & Fankhauser 2015). Intriguingly, when growing in spring, *hy5-1* silique production was strongly reduced. As *HY5* has ubiquitous functions in light response and photomorphogenesis (Cluis *et al.* 2004; Lee *et al.* 2007) the fitness effects in spring are not surprising, and our results suggest increasing importance of this transcription factor in longer photoperiods and higher light conditions.

In general, our study highlights the importance of testing gene functions *in natura*, and in addition, in varying environments. We found adverse phenotypes in these environments compared to studies in

chamber environments (e.g. flowering time in *phyA*) and could show that seasonality is important (e.g. *hy5* phenotype) for revealing potential changes in response to abiotic factors.

Acknowledgements

This chapter was performed with the help of Lucas Mohn[1] in field site preparation and maintenance of the experiment sites, Reiko Akiyama[1] supporting data analysis, and Christina Schönhuber[2] helping data collection.

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Appendix 1

Transfer of pretrated plants or seeds, or chamber condition	Cohort name	Date	Trait	Ler	*fha-1*	*fha-2*	*hy4-223N fha1*	*hy4-223 gl1-1*	*ft-2*	*ft-3*	*hy5-1*	*phyA-201*	*phyA-205*	*phyB-1*	*phyB-5*	*phyB-8*	*uvr8-1*	Applied method* ** "	Results	F- values (if applicable)	interpretation (text)
			replicates	15	15					15	16	14		14	14	13	14				
			silique number counts	9	9					7	6	10		7	10	8	9	Negative binomial GLM (no block effect), posthoc test (glht)	Global: 2.69e-06 ***, Posthoc-Test: WT/phyB <0.001 ***	Global chisq 36.054	phyB has reduced silique number in comparison with other lines, phyB differences between phyB1 and phyb5/8 (tested withposthoc glm.nb on phyb subset)
pregrown	Winter 1	14.11.14	silique number with zero	9	10					9	6	10		9	10	8	9	Negative binomial GLM (no block effect), posthoc test (glht)	Global results (anoval: class 0.0003504 ***, Posthoc-Test: WT/phyB 0.00281 **	Global chisq 24.939	phyB mutants have significantly reduced silique number compared to all other classes but hy5 (0.17) and FT (0.06), dunn.test shows significant differences against all classes
			time to flower															Kruskal-Wallis Rank Sum test, Dunn-Test	Global: 7.487e-11, Posthoc-Test: WT/phyB 0.0028 **, WT/ft 0.0013**, WT/cry2 0.0019**	Global chisq 58.911	phyB has an earlier flower time than wild type, wild type flowers earlier than ft and cry2 mutants
			survival	15	15					14	16	13		12	12	8	14	Binomial GLM	Global: 0.03715 *, Posthoc-Test: no significance, fisher: test on single genotypes reveals Ler/phyB-8 0.0131*	Global chisq 13.397	phyB-8 mutant has higher mortality
			replicates	11	6	12	16		12	12	11	12	12	12	12	12	11				
			inflorescence head counts	9	4	7	12		7	9	9	8	9	8	5	3	8	binomial GLMM, block effect is significant (1.448e-13 ***)	Global:5.934e-13 ***, Posthoc-Test: WT/hy5 <0.001 ***, WT/ft <0.001 ***, WT/cry2 <0.01 **, WT/cry1cry2 <0.01**	Global 71.958	differences between wildtype hy5 as well as late flowering ft, cry2, cry1cry2
pregrown	Winter 2	30.11.15	inflorescence head counts with zero	11	6	12	16		12	12	11	12	12	12	12	12	11	Negative binomial GLMM, block effect is significant (2.101e-07 ***, zero effect on glht prohibits testing this was. additional Kruskal-Wallis Rank Sum test, Dunn-Test	Global: 0.06095 / Kruska-Wallis-Test: 0.05018 ; Dunn-Test: WT/phyB 0.013 *, WT/ft 0.0063 **, WT/cry2 0.0152*, WT/hy5 0.0366*, WT/cry1cry2 0.0173*	Global Chisq 13.49	differences between wildtype and early flowering phyB, hy5 as well as late flowering ft, cry2, cry1cry2
			time to flower	9	4	7	12		7	9	9	8	8	8	5	3	8	Kruskal-Wallis Rank Sum test, Dunn-Test	Global: Kruska-Wallis-Test: 1.7e-09 ***, ; Dunn-Test: WT/phyB 0.0219 *, WT/ft 0.0026 **, WT/cry1cry2 0.0055 **	Global Chisq 53.894	phyB is early flowering while ft and cry1cry2 are late flowering, cry1cry2 is almost significantly later than cry2 (0.0596)
			survived	9	4	8	12		7	9	9	8	9	8	5	3	9	Poisson GLMM with block effect (p 0.00151**)	Global:0.866	Global Chisq 3.1961	no differences
			replicates	16	16	15	16		16	16	17	16	13	4	15	14	16				
			silique number counts	9	6	6	5		6	6	6	5	6	3	5	2	8	negative binomial GLMM (block effect 0.01191 *)	Global: 2.536e-10 ***, Posthoc-Test: WT/phyB <0.001 ***, WT/hy5 <0.001***	Global chisq 58.87	phyB and hy5 mutants produce less siliques than wild type Ler. Noteworthy differences between phyB mutants (tested with lmer on phyb subset)!
pregrown	Spring 1	11.05.15	silique number with zero	11	7	10	9		8	10	9	7	6	4	5	6	8	Kruskal-Wallis Rank Sum test, Dunn-Test	Global:0.01479 *; Posthoc-Test: WT/phyB 0.0049 **, WT/hy5 0.0116 *; phyB-1/phyB-8 0.0118*	Global chisq 17.436	phyB and hy5 mutants produce less siliques than wild type Ler. Noteworthy differences between phyB mutants(tested with dunn.test).
			survival	14	15	11	11		12	12	13	14	13	3	13	7	16	Negative binomial GLM (no block effect),pairwise fisher.exact	Global:0.9397	Global: 2.3245	no differences to wild type
			time to flower	14	12	15	10		12	11	10	14	13	3	14	10	16	Kruskal-Wallis Rank Sum test, Dunn-Test	Global: Kruskal-Wallis-Test: <2.2e-16 ***, Dunn-Test: WT/ft <0.001***, WT/cry2 <0.001 ***, WT/cry1cry2 <0.001 ***	Global chisq: 122.82	cry2, ft and cry1cry2 (cry2 is faster than both cry1cry2 and ft2) flower later, but phyB and uvr8 are just not earlier (p=0.0748, p=0.0918)
			replicates	8	8	8	8	8	8	8	8	8	8	8							
pregrown	Mountain (2000m)	20.06.14	time to flower	7	5	8		3	1	6		7	6	4				Binomial GLM, fishers exact test in case of significances	Global 0.688, no significance	Global chisq 4.7651	no differences in flower time
			survival	8	5	8		3	4	4		6	4	4				neg. bin. GLM	Global: 0.6309; fisher test: WT/cry1 gl1 0.0254*, WT/phyB 0.0598-	Global chisq 5.2386	cry1gl1 has lower survival, phyB almost significant

Appendix I: Details for experiments in four different environments and statistics mentioned in Chapter 1